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Prognostic role of clusterin in resected adenocarcinomas of the lung

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ABSTRACT

Rationale: Clusterin expression may change in various human malignancies, including lung cancer. Patients with resectable non-small cell lung cancer (NSCLC), including adenocarcinoma, have a poor prognosis, with a relapse rate of 30–50% within 5 years. Nuclear factor κB (Nf-κB) is an intracellular protein involved in the initiation and progression of several human cancers, including the lung.

Objectives: We investigate the role of clusterin and Nf-κB expression in predicting the prognosis of patients with early-stage surgically resected adenocarcinoma of the lung.

Findings: The level of clusterin gradually decreased from well-differentiated to poorly differentiated adenocarcinomas. Clusterin expression was significantly higher in patients with low-grade adenocarcinoma, in early-stage disease and in women. Clusterin expression was inversely related to relapse and survival in both univariate and multivariate analyses. Finally, we observed an inverse correlation between Nf-κB and clusterin.

Conclusions: Clusterin expression represents an independent prognostic factor in surgically resected lung adenocarcinoma and was proven to be a useful biomarker for fewer relapses and longer survival in patients in the early stage of disease. The inverse correlation between Nf-κB and clusterin expression confirm the previously reported role of clusterin as potent down regulator of Nf-κB.

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1. Introduction

Lung cancer is the leading cause of cancer-related mortality. Despite major advances in treatment, the prognosis for patients with lung cancer has not significantly improved in the last 2 decades [1]. When identified at an early stage, lung adenocarcinoma is primarily treated by surgical resection, which is potentially curative. However, 30–60% of patients with stage IB to IIIA non-small cell lung cancer (NSCLC) die within 5 years after surgery, primarily from recurrence of the tumor [2]. Therefore, although TNM staging is the established prognostic indicator, better parameters are

urgently needed for more accurate prediction of the outcome and more precise indication of the efficacy of treatment.

Clusterin (CLU, also known as ApoJ) is a heterodimeric glycoprotein, expressed in all tissues and found in all human fluids, including the lung, suggesting that this protein performs functions of fundamental importance both inside and outside the cell. Indeed, CLU has been found to be involved in various physiologic processes important for carcinogenesis and tumor growth, including apoptotic cell death, cell cycle regulation, DNA repair, tissue remodeling, lipid transportation, membrane recycling, and immune system regulation [3–5]. Several studies have examined the prognostic value of CLU in various malignancies, with conflicting results [6–9]. Only one study investigated the prognostic role of CLU in a heterogeneous series of resected NSCLC, and the results suggested that CLU expression could be a positive prognostic indicator for NSCLC [10].

Nf-κB is a transcription factor associated with the initiation and progression of several human tumors, including lung cancer [11] and is considered a potentially oncogenic transcription factor important for proliferation and survival of neoplastic cells [12].

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Several *in vitro* studies demonstrated that Nf- κ B activation is inhibited by CLU [13,14] and that the deletion of CLU induces activation of Nf- κ B [15] suggesting an interrelationship between these 2 proteins.

The aim of the present study was to investigate the level of expression and the prognostic role of CLU, in terms of both overall survival and disease-free survival, in a well-defined population of patients with early-stage (stages I and II), surgically resected adenocarcinoma of the lung. Furthermore, we analyzed the relationship between CLU and Nf- κ B expression and various clinical–pathological features in the same cohort of patients.

2. Methods

2.1. Patients

Files from the archives of the Section of Pathology and of the Division of Thoracic Surgery of the University of Modena and Reggio Emilia were retrospectively reviewed to retrieve all patients with a definitive diagnosis of non-small cell lung carcinoma (NSCLC) who underwent a radical surgical resection between January 2000 and December 2004. All the pathological slides were reviewed by one pathologist (G.R.) and reclassified according to the criteria set by the 2004 WHO lung tumors classification. In fact, this study was performed prior the publication of the current consensus paper [16].

Only caucasian patients with definitive diagnosis of primitive adenocarcinoma of the lung and a complete resection (R0 resection) were included; patients with other known primary tumors and a minor or incomplete resection (R1 or R2 resection) were excluded from the study. Only early stages of disease (Stage I and Stage II according to the 1997 IASLC/UICC TNM staging system for lung tumors) were considered. Eighty-three patients met all criteria and were enrolled in the study.

The percentage of bronchioloalveolar component (BAC) (as a non-replacing type adenocarcinoma according to the 2004-WHO classification) was assessed in all patients. BAC percentage was measured by counting the BAC ratio for each slide under ocular field with grid (100 grids per field) and averaging the BAC component across slides. The process was repeated twice for each slide, and the final average was used as the percentage of BAC for a given tumor case.

2.2. Immunostaining correlations

In each case, 3- μ m thick sections were obtained from a representative block. Sections were air-dried overnight at 37 °C, then deparaffinized in xylene and rehydrated through a decreasing concentration of alcohol to water. Endogenous peroxidase activity was blocked by immersion for 10 min in 3% hydrogen peroxide (H₂O₂) in methanol. Incubation with primary antibody (CLU, clone 41D, Upstate Biotechnologies, Lake Placid, NY; 1:200 dilution; microwave antigen retrieval) was accomplished with a modified streptavidin–biotin–peroxidase technique using an automated immunostainer (Benchmark, Ventana, Tucson, AZ); 3'-3-diaminobenzidine was used as the chromogene and Harris's hematoxylin as the counterstain. Negative and positive controls were included in each batch. Phospho-Nf- κ B p65 Ser536, (93H1) (Cell Signaling 1:200) was used for Nf- κ B staining, under the same conditions. Percentage of positive cells and staining intensity (negative=0, weak=1, moderate=2, strong=3) were evaluated and multiplied to render the staining score (CLU staining index and Nf- κ B staining index). In order to find the value of CLU staining index with the best accuracy in predicting prognosis, we performed a

Receiver Operating Characteristic (ROC) analysis for the CLU staining index in predicting recurrence after surgical resection (Fig. 1).

The value of 40 was associated to the cut-off value. In further survival analysis and CLU correlation, we considered a CLU staining index more than 40 as definitively positive and a value of Nf- κ B staining index higher than 0 as definitively positive.

2.3. Follow-up

Most patients were followed directly at the Division of Thoracic Surgery and Division of Respiratory Diseases of the University of Modena and Reggio Emilia with periodic office visits. Information about the remaining patients was obtained from the Oncologic Institutes treating them or by telephone interviews with the patient and/or his/her relatives. Data regarding long-term survival, presence and type of recurrence were recorded. We assigned a patient to “recurrent disease” only after histological confirmation, except for obvious clinical situations (widespread multiple lesions).

2.4. Statistical analysis

The descriptive analysis was expressed in terms of frequency, mean, median and standard deviation. Frequencies were compared with the chi-square test for categorical variables; Fischer's exact test was used for small samples. *T*-test and ANOVA were performed when comparing continuous variables. Correlation between CLU staining index and other quantitative variables was investigated with the Pearson's correlation coefficient; the goodness-of-fit measure of each linear model (*R*²) were reported. Correlation between CLU staining index and Nf- κ B staining index was investigated with the Spearman's rank correlation coefficient. Overall survival (OS) was calculated from the date of surgical resection to the date of death or last follow-up. Disease-free survival (DFS) was calculated from the date of surgical resection to the date of recurrence. The OS and DFS were calculated according to the Kaplan–Meier method. Overall Survival (OS) and DFS rates at 5 years were chosen as end points for evaluating the prognosis. Univariate analysis for any potential prognostic factor was performed using the Log-Rank test. A Cox proportional hazards model was fitted to estimate hazard ratios and confidence intervals for those prognostic factors significant in univariate analysis. A probability value (*P*) was considered statistically significant when <0.05; all *P* values were based on two-sided tests.

3. Results

The resected tumors of 83 caucasian patients with pulmonary adenocarcinoma who underwent radical resection were analyzed for CLU and Nf- κ B expression.

The clinicopathologic characteristics of the patients are reported in Table 1. Mean age for all patients was 66.0 \pm 9.1 years (range 36–84). The mean smoking index was 20.2 pack/years (range 0–60).

CLU immunostaining was considered positive in 63 patients (76%). The mean CLU staining index was 170 \pm 91.8 (median 140, range 50–270) for patient with BAC, 97.64 \pm 77.9 (median 70, range 0–270) for patients with adenocarcinoma with a variable BAC component and 41.09 \pm 62.79 (median 9, range 0–270). Nf- κ B immunostaining was considered positive in 48 patients (57.8%). The mean Nf- κ B staining index was 3 \pm 6.7 (median 0, range 0–15) for patient with BAC, 13.52 \pm 38.85 (median 0, range 0–135) for patients with adenocarcinoma with a variable BAC component and 36.43 \pm 44.29 (median 20, range 0–210).

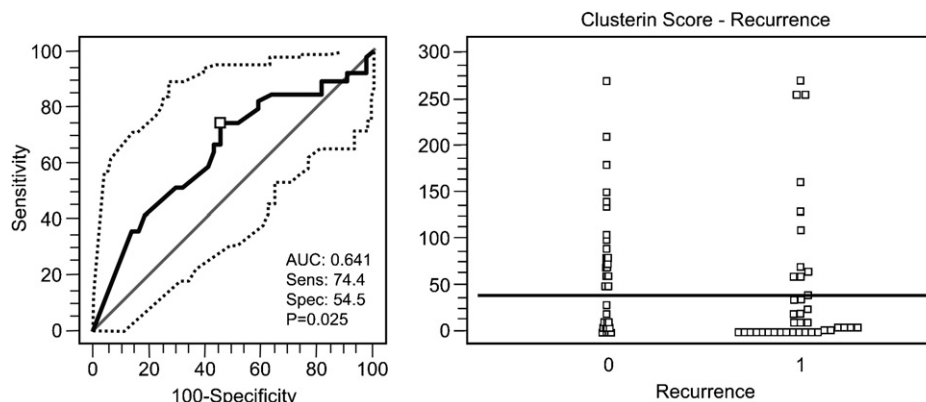


Fig. 1. Receiver operating characteristic (ROC) analysis and interactive dot diagram for the CLU staining index in relation to recurrence. The value of 40 is the ideal cutoff point to differentiate patients who experience recurrence after surgical resection (value 1) from patients who remain free of disease (value 0).

3.1. CLU immunostaining correlations

The correlations between immunohistochemical expression of CLU and Nf-kB with clinical–pathological variables are reported in Table 2.

A significant association was noted between CLU expression and sex ($P=0.024$), histological grading ($P<0.001$), presence of BAC component ($P=0.002$) and pathologic stage ($P=0.016$). CLU was expressed significantly more often in women, although smoking habit and age were not statistically significant between males and females, in low-grade adenocarcinoma and in early stages of cancer progression. In particular, all pure BAC tumors significantly expressed CLU, whereas poorly differentiated adenocarcinomas frequently did not express CLU.

Nf-kB was significantly more expressed in high-grade ($P=0.034$) and invasive adenocarcinomas without BAC component ($P=0.028$). Therefore, a highly significant inverse correlation was found

between CLU and Nf-kB expression: high levels of CLU expression were associated with low levels of Nf-kB expression (Spearman's $\rho = -0.4282$, $P=0.0001$) (Fig. 2).

To better clarify which clinical–pathologic parameters significantly correlated with CLU expression, we also performed a bivariate statistical analysis. Tumors that expressed a BAC component (pure BAC, and mixed adenocarcinoma with BAC component) were correlated with CLU staining index, and a highly significant linear correlation with the percentage of BAC component was found. The CLU staining index increased significantly with the percentage of BAC component (Pearson correlation coefficient 0.726; $R^2 = 0.527$; $P=0.001$).

In the whole series, an inverse linear correlation between CLU and smoking index approached significance (Pearson correlation coefficient -0.215 ; $R^2 = 0.046$; $P=0.067$). Conversely, no significant correlation was found between the CLU staining index and age (Pearson correlation coefficient -0.086 ; $R^2 = 0.007$; $P=0.440$) or tumor size (Pearson correlation coefficient -0.025 ; $R^2 = 0.001$; $P=0.823$).

Table 1
Patients' characteristics.

Variable	No. (%)
Sex	
Male	62 (74)
Female	21 (26)
Smoking history	
Yes	66 (79)
No	17 (21)
Type of resection	
Lobectomy	77 (93)
Bilobectomy	2 (2)
Pneumectomy	4 (5)
Pathologic stage	
IA	26 (31)
IB	42 (50)
IIA	3 (4)
IIB	12 (15)
Grading	
G1	12 (14)
G2	20 (24)
G3	51 (62)
Adenocarcinoma subtype	
Acinar	38 (46)
Mixed	
With BAC component	13 (18)
Other mixed	4 (4)
Papillar	8 (10)
Solid with mucin production	8 (10)
Mucinous "colloid"	3 (3)
Signet ring	2 (2)
Fetal type	2 (2)
Clear cell	1 (1)
Pure BAC	4 (4)

3.2. Analysis of survival

Follow-up was discontinued for all patients in July 2008. Median follow-up was 44.5 months (3.7 years) (range 1–97 months). Overall 5-year survival rate was 50% with a median survival time of 61 months. Eight deaths were not cancer related; 33 patients died of the disease. The rate of DFS was 48% with a median disease-free survival time of 55 months. The tumors recurred in 38 patients (47.4%); 13 patients with intrathoracic recurrence and 25 patients with systemic dissemination. Six patients were alive with recurrent disease at the end of follow-up. A significant correlation was found between the crude recurrence rate and the CLU staining index: 29.4% for positive CLU expression and 59.2% for negative CLU expression; $P=0.008$.

Results of univariate analysis regarding OS and DFS for the clinicopathologic prognostic factors are shown in Table 3. Stage, grading, and CLU staining index predicted both OS and DFS, while sex predicted only overall survival. Nf-kB staining index did not predict OS neither DFS. Regarding the CLU staining index, univariate survival analysis showed a 5-year OS rate of 72% for patients with positive CLU expression and 35% for patients with negative CLU expression (log rank 9.92; $P=0.001$) and a 5-year DFS rate of 67% for patients with positive CLU expression and 34% for patients with negative CLU expression (log rank 8.49; $P=0.003$).

When we stratified the analysis of survival by pathologic stage, the CLU staining index remained a significant prognostic factor even at early stages (IA and IB): a 5-year OS rate of 73% for

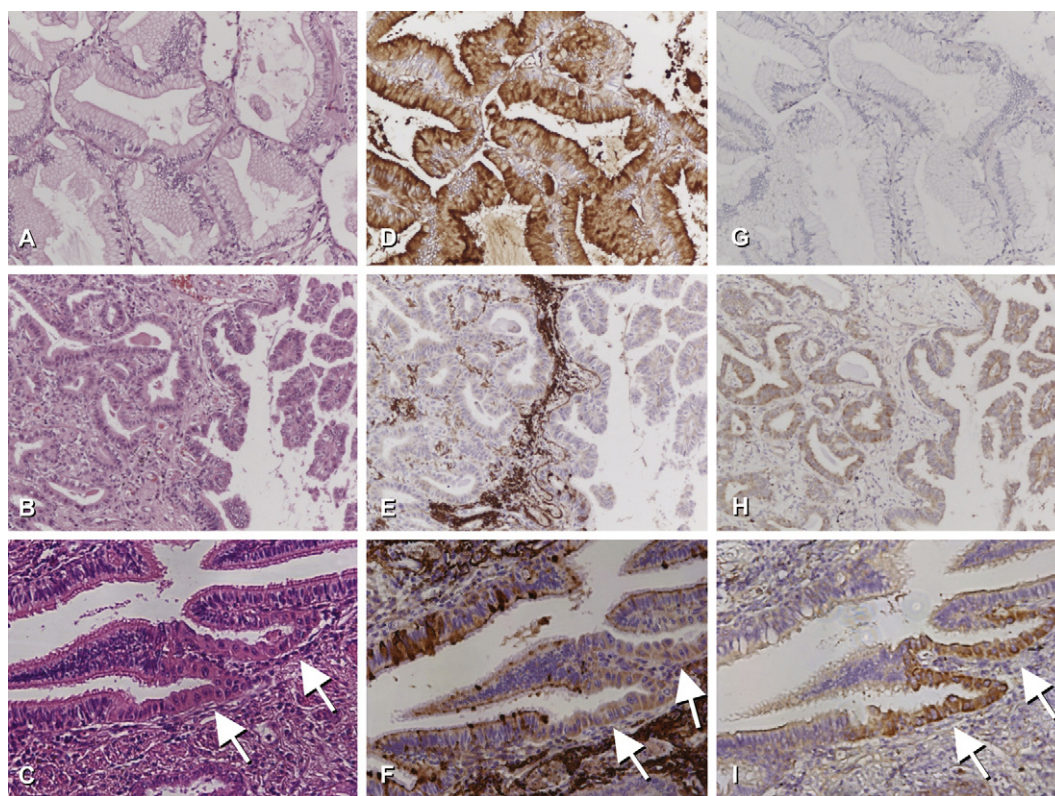


Fig. 2. Hematoxylin–eosin staining, clusterin staining and Nf-kB staining in a pure mucinous-type bronchioloalveolar carcinoma (A, D and G, respectively), in a papillary adenocarcinoma (B, E and H, respectively) and in partial colonization of a normal bronchiole by 2 foci of well-differentiated adenocarcinoma (see arrows) (C, F and I, respectively).

CLU-positive patients vs. 42% for CLU-negative patients ($P=0.016$), and a 5-year DFS rate of 66% for CLU-positive patients vs. 39% for CLU-negative patients ($P=0.021$).

To evaluate the independent prognostic value of CLU expression, we performed a multivariate analysis using the Cox proportional hazards model with the variables significantly related to survival by univariate analysis (Table 4). The CLU staining index was found to be the only independent factor predicting recurrence, whereas pathologic stage was the only prognostic factor significantly associated with OS. A positive CLU staining index score was significantly associated with longer DFS ($P=0.048$); the association with OS approached statistical significance ($P=0.052$). Adenocarcinomas with a negative CLU staining index score had a relative risk of

recurrence of 2.18 (95% confidence interval 1.00–4.75) compared to CLU-positive tumors; patients with adenocarcinoma had a relative risk of death of 2.39 (95% confidence interval 0.99–5.77). Early-stage disease (IA and IB) predicted greater survival than stage II disease ($P=0.02$). Neither tumor differentiation nor sex had any significant association with death or recurrence in multivariate analysis.

4. Discussion

To our knowledge, this is the first study exploring *in vivo* the existence of a correlation between Nf-kB and CLU expression in lung cancer.

Table 2

Association between clusterin staining index and Nf-kB staining index and clinicopathologic variables.

Variable	Clusterin staining		<i>P</i>	Nf-kB staining		<i>P</i>
	Positive ^a	Negative ^a		Positive ^a	Negative ^a	
Sex						
Male	21 (34%)	41 (66%)	0.024	37 (60%)	25 (40%)	0.615
Female	13 (62%)	8 (38%)		11 (52%)	10 (48%)	
Grading						
G1	10 (83%)	2 (17%)	0.001	4 (%)	8 (%)	0.034
G2	11 (55%)	9 (45%)		9 (%)	11 (%)	
G3	13 (25%)	38 (75%)		35 (%)	16 (%)	
Histological subtype						
BAC	4 (100%)	0	0.002	1 (25%)	3 (75%)	0.028
ADK-BAC	9 (69%)	4 (31%)		4 (31%)	9 (69%)	
Other subtypes	21 (32%)	45 (68%)		43 (65%)	23 (35%)	
Pathologic stage						
IA + IB	32 (47%)	36 (53%)	0.016	40 (83%)	8 (17%)	0.776
IIA + IIB	2 (13%)	13 (87%)		8 (53%)	7 (47%)	

Abbreviation: ADK-BAC, adenocarcinoma with BAC component.

^a Raw percentages.

Table 3
Results of univariate analysis of overall survival and disease-free survival.

Variable	Overall survival		Disease-free survival	
	5 years (%)	P value	5 years (%)	P value
Sex				
Male	42	0.013	42	0.118
Female	71		62	
Grading				
G1	83	0.026	90	0.016
G2	58		54	
G3	37		35	
Histological subtype				
BAC + ADK-BAC	57	0.158	61	0.159
Other subtypes	45		45	
Pathologic stage				
IA	79	0.001	36	0.024
IB	42		21	
IIA + IIB	28			
CLU staining index				
Positive	72	0.001	67	0.003
Negative	35		34	
NFKB staining index				
Positive	45	0.990	42	0.662
Negative	52		55	

Abbreviation: ADK-BAC, adenocarcinoma with BAC component.

Our data suggest that CLU expression is an independent prognostic factor in surgically resected adenocarcinoma of the lung. Multivariate analysis showed that low CLU expression is significantly related to a high rate of recurrence and death after surgical resection. Given the established prognostic role of pathologic stage in lung cancer, we also performed a subgroup analysis stratified for disease stage: patients with stage I disease who had high levels of CLU expression showed significantly higher OS and DFS with respect to patients with the same stage with low levels of CLU.

The highly significant inverse correlation found between CLU and Nf-κB expression is consistent with what is already known about the inhibitory activity of CLU on Nf-κB activity and expression [15]. However, although Nf-κB is known to be involved in initiation and cancer progression, there are no studies showing a correlation between Nf-κB and cancer OS and DFS.

In a recent study, Dimitrakopoulos and colleagues showed that cytoplasmic immunoreactivity of NF-κB2 and RelB was correlated with tumor stage. In addition, cytoplasmic NF-κB2 levels were also related to tumor grade and expression of RelB in the cytoplasm was tumor histologic type-specific, with squamous cell carcinomas having the highest protein levels [17]. In contrast, in our study CLU serves as a biomarker for OS and DFS.

Similar findings were reported by Albert et al., who investigated CLU expression levels in patients with different histologic types of NSCLC [10]. In contrast, we focused our study on early-stage (stages I and II) adenocarcinoma in order to investigate the possible involvement of CLU in early stages of cancer progression.

Table 4
Results of multivariate analysis (Cox proportional Hazard Models).

Variable	Death		Recurrence	
	HR (95% CI)	P value	HR (95% CI)	P value
CLU				
Negative vs. positive	2.39 (0.99–5.77)	0.052	2.18 (1.00–4.75)	0.048
Grading				
G3 vs. G1 + G2	1.40 (0.64–3.07)	0.393	1.59 (0.73–3.48)	0.237
Pathologic stage				
IIA + IIB vs. IA + IB	2.26 (1.10–4.65)	0.026	1.19 (0.52–2.68)	0.675
Sex				
Male vs. female	2.02 (0.92–4.40)	0.077	1.62 (0.74–3.58)	0.221

Abbreviations: HR, hazard ratio; CI, confidence interval.

In addition, this is the only histologic type of lung cancer whose incidence is increasing, according to epidemiological studies [18]. Moreover, our series included a select group of patients who were racially homogeneous, being all caucasian, because of the different characteristics of adenocarcinomas [19].

The prognostic and predictive [20] value of CLU has already been evaluated in various malignancies, but conflicting results have been reported [6–9,21–24]. Conflicting data on the levels of CLU in lung cancer have also been found. *In vitro* and *in vivo* studies have reported the presence of high levels of CLU in lung cancer cells [25]. However, data available on the Oncomine database showed that CLU was downregulated in tumors as compared to normal lung (www.oncomine.org). Downregulation of CLU, and a further reduction of its expression during cancer progression, can also be gleaned from the results of the present study. CLU expression significantly decreased from well-differentiated to poorly differentiated adenocarcinoma, and from stage I to stage II. This data and the survival analyses confirm the positive prognostic role of CLU in lung adenocarcinoma. The earlier findings of high levels of CLU in lung cancer cells [25] is not contradictory with our experimental data, because CLU was indeed positively expressed in 41% of adenocarcinomas from our select population.

Here we investigated for the first time the correlation between CLU expression and adenocarcinoma subtypes, in particular those that express a BAC component. We found BAC in only four patients (4.8%). However, up to 20% of invasive adenocarcinomas of the lung showed a mixed pattern, ranging from predominant BAC with a small focus of invasion, to invasive adenocarcinoma with an isolated group of cells with BAC features [18,26]. In our series, mixed adenocarcinomas with a BAC component accounted for 16% of all adenocarcinomas. In cases with pure BAC, CLU was expressed at high levels, whereas only 30% of cases of invasive adenocarcinoma expressed high levels of CLU. In mixed adenocarcinomas, with a variable amount of BAC component, CLU expression was intermediate, being present in 69% of cases; furthermore, CLU expression was directly related to the amount of BAC component, decreasing with the increased invasiveness of the lesions. The molecular events that regulate the multistep carcinogenesis – hypothesized in lung adenocarcinoma – are currently unknown, but multiple pathways and genetic changes are certainly implicated [27–31]. The progressive decrease of CLU expression is clearly one of the molecular changes implicated in the progression from pure BAC to invasive adenocarcinoma, and confirm the possible role of CLU as a tumor suppressor factor against infiltration or invasiveness. In fact, CLU has been already found as an important regulator of cell fate, negatively controlling further progression and invasiveness [12,32].

Tang et al. [11] showed that Nf-κB expression is an early and frequent feature of lung cancer. Furthermore, Nf-κB expression level was significantly higher in advanced adenocarcinomas of the lung compared to earlier stages [12].

Inverse linear correlation of CLU and Nf-κB expression is consistent with *in vitro* studies demonstrating and important regulatory

loop between these two genes, i.e. while CLU is one of the most highly regulated gene by Nf-kB, CLU also regulates Nf-kB activity in a negative manner by stabilizing I-kBs. These data led to the hypothesis that CLU participates in a negative loop in which transcriptional activation of CLU is evoked to dampen Nf-kB activity [14].

In conclusion, CLU expression is an independent positive prognostic factor in surgically resected adenocarcinoma of the lung. CLU expression could therefore be used as a novel biomarker for identifying patients with stage IB adenocarcinoma who require chemotherapeutic treatment after surgical resection. The progressive decrease in CLU expression from well-differentiated to poorly differentiated adenocarcinomas, along with the oncogenic progression from pure BAC, to mixed adenocarcinoma with BAC component, to invasive carcinoma, confirm a positive prognostic role for CLU in patients with lung adenocarcinoma. Finally, we observed an inverse correlation between Nf-kB and CLU, thus providing new insights into the potential role in pathogenesis of these proteins during lung cancer progression and final outcome.

Conflict of interest

None declared.

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